

Detection of clinically relevant yeast in blood: evaluation of methods for RNA extraction and amplification

Detecção de leveduras clinicamente relevantes no sangue: avaliação de métodos de extração e amplificação de RNA

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ABSTRACT

Objectives: To evaluate different methods of extraction and purification of RNA from yeast in blood samples to be used in molecular-based diagnosis. **Methods:** Different combinations of commercially available kits were tested with blood samples artificially inoculated with yeast cultures. **Results:** Qiagen kits performed better when compared to other commercial kits both for RNA extraction and one-step RT-PCR. **Conclusions:** Kits using a hot-start approach are better; the choice of kits has an important impact on the final result of a test.

Keywords: Yeast, molecular-based diagnosis, RNA extraction, RT-PCR

RESUMO

Objetivos: Avaliar diferentes métodos de extração e purificação de RNA de leveduras em amostras de sangue para ser usado em diagnóstico molecular. **Métodos:** Diferentes combinações de kits comerciais foram testadas com amostras de sangue artificialmente inoculadas com cultura de levedura. **Resultados:** Os kits da Qiagen apresentaram os melhores resultados quando comparados com os demais kits comerciais, tanto para extração quanto para a RT-PCR em etapa única. **Conclusões:** Kits que utilizam "hot-start" são melhores e a escolha do kit adequado tem impacto importante no resultado final de um teste.

Descritores: Levedura, diagnóstico molecular, extração de RNA, RT-PCR

INTRODUCTION

Advances in Medicine have led to extended survival of immunocompromised patients and increased importance of opportunistic fungal infections⁽¹⁻²⁾. *Candida* is the most common opportunistic yeast, causing severe infections characterized by high mortality rates. Antifungal agents are particularly toxic to human cells and the use of such drugs must comply with strict criteria. Nevertheless, early and aggressive antifungal therapy taking into account the etiologic agent involved could improve prognosis⁽³⁻⁴⁾. Conventional laboratory techniques for yeast identification (culture, biochemical, physiological and morphological features) have a prolonged turnaround time. Faster and more sensitive molecular-based diagnostic tools are valuable in this setting. Higher sensitivity can be achieved by using ribosomal RNA as template in amplification assays, since it encompasses 90% of the total RNA in cells. By using RNA as a target, it is necessary to first reverse transcribe this RNA into cDNA (complementary DNA), which is then used as template for an amplification approach (PCR). To achieve consistent results, inhibitory substances that interfere with these techniques and are commonly present in biological samples are eliminated. Hence, extraction and purification of nucleic acids are fundamental issues in molecular-based diagnosis.

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Supported by FAPESP 00/14545-9.

Received on May 10, 2004 – Accepted on August 12, 2004

In this paper, we compare different methods for RNA extraction from yeast cells spiked in blood and different reagents for enzymatic amplification. Published protocols to obtain protoplasts and four commercially available RNA extractions kits were evaluated. The extracted RNA of each procedure was assayed in a one step RT-PCR format using four kits and a set of primers directed to 18S rRNA. This approach was applied to five different species of *Candida* artificially inoculated in blood.

METHODS

Strains and growth conditions

Yeasts were cultivated in YPD (0.5% yeast extract, 1% peptone and 2% glucose) at 30°C and 150 rpm. *Candida albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis* were isolated from patients and identified by the Clinical Laboratory by the Vitek system and confirmed by chromogenic medium (CHROMOagar, Probac) and by 18S sequencing. The manufacturer's (DYEnamic ET Terminator Cycle Sequencing kit – Amersham Biosciences) recommendations were followed during sequencing and used in the primers described⁽⁵⁾.

Spiking blood samples

Culture negative blood samples that would be discarded, were kindly provided by the Clinical Laboratory, with no patient identification. Blood was inoculated with different concentrations of cultivated yeast estimated by spectrophotometer (600 nm) and cell count in a Neubauer chamber.

Protoplast preparation from spiked blood samples

Blood cells were disrupted in lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.05% proteinase K and 0.05% Tween 20) at 55°C, for 30 min. Debris were washed with lysis buffer without proteinase K and centrifugation at 10.000 g, for 8 minutes, at 20°C until clarification of the samples. Consecutive treatment of the remaining suspension with NaOH at 95°C, for 10 minutes, followed by one hour at 37°C, with 0.1% Zymoliase and 1% b-mercaptoethanol solution were used for protoplast preparation.

RNA extraction from protoplasts and RT-PCR

We strictly followed the recommendations of the different RNA extraction kits: Purescript Yeast and Gram-Positive Bacteria Kit (Gentra), "High Pure RNA

Isolation Kit" (Roche), "RNeasy Kit" (Qiagen) e Trizol (Invitrogen). Gentra's kit utilizes a detergent to disrupt protoplasts, whereas the other kits use a chaotropic agent, a guanidine salt. For the purification step, Gentra uses salting out, and Trizol has phenol in its composition. The other two kits have some minor variations but both use columns to purify the RNA. Qiagen's column excludes small-sized RNA, with less than 200 bases, eliminating 15 to 20% of 5.8S and tRNA. We also tested RT-PCRs kits from Invitrogen (SuperScript One-Step RT-PCR with Platinum *Taq*), Applied Biosystems (EZ *rTth* RNA PCR Kit), Qiagen (Qiagen OneStep RT-PCR) and Roche (C. Therm. Polymerase One-Step RT-PCR System). Primers and PCR cycling conditions have been described⁽⁵⁾. We used 30 pmol of each primer in 50 µl volume RT-PCR. Regular PCR was also performed in order to check for the absence of DNA in the extracted RNA samples.

RESULTS

Since a diagnostic test aims to achieve good sensitivity and reproducibility, different methods for obtaining and amplifying nucleic acids from pathogens in clinical samples were evaluated. Blood samples artificially inoculated with yeast cultures were submitted to two previously described protocols to discard human cells and to generate fungal protoplasts⁽⁵⁻⁶⁾. The protoplasts obtained were further disrupted and DNA was precipitated and re-suspended in water. Growth curves and counting of yeast cells in a Neubauer chamber equalized yeast cell cultures before analysis.

After obtaining protoplasts, the four commercial kits were evaluated for RNA extraction. Although Qiagen's protocol claims to eliminate DNA through the column and Roche's assay includes a DNase step, all samples extracted with any of the four kits showed a positive result upon regular PCR, indicating the presence of residual DNA in the samples.

The extracted fungal RNA obtained with each of the extraction kits was then used in reverse transcription and amplification using four different commercial kits. Qiagen kit has two reverse transcriptases that present specific activity to low and high RNA concentration. The DNA polymerase used for amplification is a hot-start enzyme, that is, it is inactive during the transcription step. Invitrogen kit also has a hot-start enzyme, a Platinum *Taq* polymerase, which has its active site blocked by an antibody during the processing of RNA. A thermostable polymerase is used in the reverse transcription step of Roche kit, followed by the typical PCR polymerization guided by a second enzyme present in the reaction. The fourth kit tested was purchased by

Applied Biosystems and utilizes a single enzyme that performs both reverse transcription and amplification. All assays, including the protocols to obtain protoplasts, were performed in duplicates to assess reproducibility and the results are presented in Table 1. RNA extracted with Gentra kit did not show amplification in any of the RT-PCR assay, although the positive control was amplified (pure yeast culture), and the results were not included in the Table. Qiagen RT-PCR was the only kit that succeeds in amplifying all samples, independently of the RNA extraction method used.

Table 1. Comparison of different combinations of extraction and RT-PCR kits to be used in fungal diagnosis

Isolates	Extraction Kits	RT-PCR kits			
		INVITROGEN RT-PCR	APPLIED RT-PCR	QIAGEN RT-PCR	ROCHE RT-PCR
CA	QIAGEN	+/+*	-/+	+/+	-/-
	ROCHE	-/-	-/-	+/+	-/-
	TRIZOL	+/+	-/-	+/+	-/-
CG	QIAGEN	+/+	-/-	+/+	+/-
	ROCHE	-/-	-/-	+/+	-/-
	TRIZOL	+/-	+/+	+/+	-/-
CK	QIAGEN	+/+	-/-	+/+	-/-
	ROCHE	+/-	-/-	+/+	-/-
	TRIZOL	+/+	-/-	+/+	-/-
CP	QIAGEN	+/+	-/-	+/+	-/-
	ROCHE	-/-	-/-	+/+	-/-
	TRIZOL	-/-	-/-	+/+	-/-
CT	QIAGEN	+/+	-/-	+/+	-/-
	ROCHE	-/-	-/-	+/+	-/-
	TRIZOL	-/+	-/-	+/+	-/-

*Results are presented in duplicates. CA, *C. albicans*; CG, *C. glabrata*; CK, *C. kruzei*; CP, *C. parapsilosis*; CT, *C. tropicalis*

DISCUSSION

The importance of obtaining reproducible data for clinical diagnosis of infectious diseases led several authors to compare different molecular-based approaches⁽⁷⁻⁸⁾. In this study, we compared procedures for extraction and amplification of rRNA (ribosomal RNA) from different *Candida* species. For protoplast production there is no commercial kit available and two previously described in-house approaches were tested⁽⁵⁻⁶⁾. A third method resulting from the combination of the two approaches yielded the best results, including the ease of manipulation. Modifications in the protocol combined elimination of human cells by enzymatic lysis, as described by Fujita⁽⁶⁾, and treatment of yeast cells with NaOH at 95° to improve protoplasts yield, as suggested by Einsele⁽⁵⁾.

RNA extraction and RT-PCR were also tested using commercially available kits. A one-step approach for reverse transcription and amplification in one tube was preferred to avoid opening tubes during the process. The use of a single tube to reverse transcribe and amplify was

preferred since it minimizes the risk of cross-contamination. Moreover, the one-tube RT-PCR format can be used with a real-time amplification technology with advantages over other methods, such as improved sensitivity and shorter turnaround time. This is particularly important when dealing with ubiquitous organisms, such as *Candida* and universal panfungal primers as those directed to conserved regions as rRNA, a putative source of cross-contamination⁽⁹⁾.

Our results suggest that kits using a hot-start approach are better than those that allow polymerization at lower temperatures. This feature is especially important in RNA quantification assays, when the initial number of templates (cDNA) must correspond to the actual RNA load⁽¹⁰⁾.

CONCLUSIONS

In general, kits using a hot-start approach (Invitrogen and Qiagen) had better performance than those that allow polymerase to start its action during the reverse transcription step. Our results showed a better performance of Qiagen kit both for RNA extraction and RT-PCR assay and showed the importance of choosing an appropriate kit for molecular diagnosis.

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